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Interactions between arbuscular mycorrhizal fungi, rhizobacteria, soil phosphorus and plant cytokinin deficiency change the root morphology, yield and quality of tobacco

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) and rhizobacteria (*Pseudomonas fluorescens*) have been targeted for plant growth promotion in order to reduce agrochemical inputs. However, the effects of their interaction on root morphology under different nutrient levels are unknown. Moreover, both soil microorganisms can alter the levels of plant hormonal regulators but no *in vivo* evidence is available for their interplay with cytokinin (CK) on root morphology. In a full-factorial greenhouse experiment we investigated the effects of interaction between *Rhizophagus intraradices*, *P. fluorescens 8569r*, phosphorus (P) amendment and plant CK deficiency on tobacco root morphology, shoot yield and quality. Our results suggest that reduced CK levels may be involved in plant signaling to stimulate AMF hyphal growth in the roots. In addition, we document that a bacteria isolated from the rhizosphere of a non-mycorrhizal plant can function as mycorrhizal helper bacteria, most likely via interplay with phytohormones. The two soil microorganisms, depending on the inoculum combination, P amendment and plant CK levels, modified tobacco root morphology. Our results suggest that the positive interactions between *P. fluorescens* and AMF depend on soil nutrient status and root hormonal balance. Both microorganism modified shoot yield and these effects seem to result from altered root morphology. Overall, our study support early conclusions that the classification of a soil microorganism as detrimental or beneficial should be based upon their net effects on the plant growth according to circumstances. We suggest this consideration to be extended to the effects of interaction between soil microorganisms on root morphology.

1. Introduction

Modern agriculture relies on high input of agrochemicals which cause major environmental problems (Spiertz, 2010). Feeding an increasing human population and reducing the impacts on the environment urges for low input agricultural practices. Some argue that the solution can be found belowground in the rhizosphere (Gewin, 2010). There is still room for improvement by better exploring the genetic variability of roots to improve root morphology, their ability to forage or fixate nutrients or by manipulating soil microbes which improve the root function (Gewin, 2010).

Essentially, the root system is responsible for the acquisition of water and nutrients from the soil. Due to soil heterogeneity, a root morphology adapted to the soil environment is critical for plant survival and yield (Malamy, 2005). The morphological responses of roots to soil abiotic factors such as mineral nutrients have been well documented and reviewed (e.g. Forde and Lorenzo, 2001; Lopez-Bucio et al., 2003). However, our understanding of the effects of multiple biotic interactions belowground on root morphology is still in its infancy. This is particularly important when considering the development of microbial inoculations as a solution to improve plant growth and reduce chemical inputs.

Among the soil organisms targeted for plant growth promotion, the ubiquitous arbuscular mycorrhizal fungi (AMF) and *Pseudomonas fluorescens* have received considerable attention (e.g. Lugtenberg and Kamilova, 2009; Smith and Smith, 2011a). AMF can promote the growth of plants by enhancing the uptake of nutrients, in particular phosphorus (P) (Smith and Smith, 2011a). In addition, AMF can alter root morphology in most studies by inducing a more profusely root branching (Berta et al., 1993, 2005; Gamalero et al., 2002; Gamalero et al., 2004; Gutjahr et al., 2009; Olah et al., 2005). In contrast to AMF, changes in root morphology induced by *P. fluorescens* are not so often reported; most studies focus on the enhancement of root growth and length (Dhillion, 1992; Gamalero

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et al., 2003; Germida and Walley, 1996; Neeraj and Singh, 2011) with fewer showing positive or neutral effects on root surface area, volume and branching (Berta et al., 2005; Gamalero et al., 2002, 2004). To the extent of our knowledge, only one study has addressed the effects of interactions between *P. fluorescens* and AMF on root morphology, showing these to be either neutral or synergistic (Gamalero et al., 2004). However, the soil microorganisms are not always beneficial and the function of AMF and *P. fluorescens* may change depending on the edaphic conditions, such as nutrient availability and microbial densities (Alström, 1987; Gamalero et al., 2002; Hoeksema et al., 2010; Johnson et al., 1997). This raises the question if the effects of this biotic interaction on root morphology could be modified by abiotic factors such as fertilization, in particular with P.

Furthermore, the biotic and abiotic regulators of root morphology are both part of the responsive pathway of root morphogenesis (Malamy, 2005). Ultimately, it is the intrinsic pathways, i.e. the genetic and hormonal regulators, which modulate organogenesis and growth and determine the characteristic morphology of the root as induced by the responsive pathway (Malamy, 2005). Cytokinin (CK) is an important intrinsic, negative regulator of root growth and elongation and a systemic signaling of plant P starvation (Franco-Zorrilla et al., 2005; Werner et al., 2003, 2001). AMF colonization can alter the CK levels in the roots, irrespective of the improvement of P nutrition, which may result from indirect modification of plant hormonal production or directly by fungal CK synthesis in the root (Shaul-Keinan et al., 2002; Torelli et al., 2000). Moreover, it seems likely that CK may be involved in the AMF alteration of root morphology, but no experimental evidence exists supporting this mechanism. The enhancement of root length by *P. fluorescens* have been associated to bacterial synthesize of auxin (Gamalero et al., 2003, 2004), and the later is known to act in conjunction with CK to modulate root morphogenesis (Werner and Schmülling, 2009).

To further elucidate the relations between intrinsic and extrinsic abiotic and biotic regulators of root morphology, we have focused on the interaction among *Rhizophagus intraradices*, *P. fluorescens*, P amendment and plant CK levels, by comparing a tobacco wild type (WT) to a CK-deficient line. Furthermore, in agricultural systems the root efficiency in N uptake is of high importance for crop production (Spiertz, 2010). This is particularly important for tobacco, whose leaf yield and nicotine, an important chemical trait of tobacco quality, are strongly affected by N availability (Fritz et al., 2006; Ruiz et al., 2006; Russell et al., 1980; Steppuhn and Baldwin, 2007). Therefore, we evaluated as well the competence of root morphological changes induced by the biotic and abiotic interactions in affecting tobacco shoot yield and concentrations of N and nicotine in leaves.

2. Materials and methods

2.1. Plant lines and soil microorganisms

As experimental plants we used two lines of tobacco (*Nicotiana tabacum* L. cv. Samsun NN), the WT and the *35S:CKX2* transgenic line. The latter was developed and reported by Werner et al. (2001). Shortly, the *35S:CKX2* line was constructed by cloning a gene encoding a protein with cytokinin oxidase (*CKX*) activity, the *AtCKX2* gene from *Arabidopsis thaliana*. The gene was positioned in the tobacco WT under the control of a constitutive 35S promoter. The line *35S:CKX2* overexpress *CKX* and, consequently, has reduced endogenous concentrations of CK (Werner et al., 2001).

As AMF species we used *R. intraradices* (Walker & Schüßler; basionym *Glomus intraradices* Schenck & Smith), which is known to colonize the roots of *N. tabacum* L. cv. Samsun NN (Herrera-Medina et al., 2003). The inoculum of AMF was produced from *in vitro* cultures (MUCL 43204, GINCO, Belgium) and consisted of an aseptic suspension of spores, hyphal fragments and Ri TDNA transformed carrot roots heavily colonized. To produce a mock solution for the non-AMF controls, part of the supernatant of the AMF inoculum was filtered through a sterile 0.22 μm filter, carrying only potential compounds dissolved in the solution.

The *P. fluorescens* Migula (strain 8569, DSMZ, Germany) was isolated in Germany from the rhizosphere of rapeseed (*Brassica napus* L.), a non-mycorrhizal plant species (Glenn et al., 1985), and can function as plant growth promoting rhizobacteria (PGPR) (Kristek et al., 2008). To selectively culture the bacterium at the end of the experiment, we developed a strain of *P. fluorescens* resistant to antibiotics with a method adapted from Gamalero et al. (2004). This was done using a stepwise concentration technique on Caso Agar enriched with rifampicin (Sigma, R3501) until a concentration of 100 mg ml^{-1}. The spontaneously resistant mutant was designated as *P. fluorescens 8569r*. After culturing, the bacterium was scraped from the medium into a sterile buffer solution (0.1 M $MgSO_4 \cdot 7H_2O$). The density was then determined and adjusted to 6×10^9 colony forming units (CFU) per ml. To produce a mock solution for the non-*P. fluorescens 8569r* controls half of the *P. fluorescens 8569r* suspension was autoclaved (20 min at 121 °C).

2.2. P amendments

Two modified Hoagland's solutions enriched with iron were produced to supplement the plants with two levels of P amendments, low (8 mg l^{-1}) and high (16 mg l^{-1}). The macronutrients concentrations in the low and high P amendments were adapted from the solutions No 5 and 7 described by Douds and Schenck (1990), respectively. The iron supplement followed the recommendation by Smith et al. (1983) for sand cultures. 30 ml of each nutrient solution were added every second day according to treatment (described in Section 2.3).

2.3. Treatments and growth conditions

A full factorial experiment was set up in the greenhouse (16 h light and 23°/28 °C night/day temperatures), with the tobacco WT or the *35S:CKX2* transgenic line, with presence or absence of AMF or *P. fluorescens 8569r*, and with low or high P amendment. The experiment had in total 128 experimental units (containers) divided by 16 treatments, each treatment with 8 replicates. The 500 ml containers were previously sterilized in bleach for 20 min, washed with tap water, and received each a cotton mesh inserted at the bottom to prevent substrate losses. Sand (CEMEX GmbH, Kraatz, Germany) was autoclaved at 121 °C for 20 min to eliminate potential presence of microbes, and then distributed into the containers (ca. 600 g of sand per container). Using a sterilized dibber, a hole was made on the sand surface in each container to add the microorganism inocula. 64 containers received each 1 ml of AMF inoculum solution containing spores (50–100), hyphal fragments and colonized root fragments. The amount of 50 spores of *R. intraradices* per plant has been shown adequate to cause considerable mycorrhizal colonization in tobacco plants (about 65%) after 50 days of inoculation (Herrera-Medina et al., 2003). The other 64 containers received each 1 ml of the AMF mock solution. According to Hinsinger et al. (2009), a single gram of soil can contain 10^7–10^{12} bacteria. To re-populate the 600 g of sterile sand with similar levels of our target soil bacteria, half of the containers treated with AMF inoculum received 1 ml of living *P. fluorescens 8569r* suspension (6×10^9 CFU), while the other half received 1 ml of bacterial mock suspension. The 64 containers treated with AMF mock solution received similar inoculations with *P. fluorescens 8569r* suspension or mock suspension. Each container was placed